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MULTIPLE ACTIONS OF ADENOSINE 5′-TRIPHOSPHATE ON CHICK SKELETAL MUSCLE

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SUMMARY

1. Extracellularly applied adenosine 5′-triphosphate (ATP) is known to have an excitatory action on chick skeletal muscle. By making intracellular recordings from cultured chick myotubes bathed with blockers of several types of voltage-dependent channels, the direct action of ATP could be observed.

2. When muscle cells were studied near their resting potential, ATP usually produced a biphasic response. There was a rapid initial depolarization, followed by a slower repolarization. The repolarization could drive cells negative to their initial resting potential, indicating that it was not due simply to desensitization of the process that produced the depolarization. Thus there are at least two distinct responses to ATP.

3. At room temperature the early response to ATP activated within 20 ms, and the second response activated with a latency of approximately 1 s. In our standard blocking solution, the average reversal potential of the early response was −17 mV, while the late response had a reversal potential that was negative to −70 mV. In a few cells the second response appeared to be absent.

4. The amplitude and time course of the late response were substantially decreased by low temperature (12 °C) and increased by high temperature (37 °C). In contrast, temperature had much smaller effects on the early response. Both the time course and temperature dependence of the late response suggest that an intracellular second messenger system may be involved in its activation.

5. Ion-substitution experiments were performed to determine the type of conductance changes that evoke each response. These indicated that the early response was due to an increased membrane permeability to sodium, potassium and chloride, but not to large cations or anions, and that the late response was due to an increased permeability to potassium.

6. Measurement of the responses of muscle cells to acetylcholine supported the conclusion that both anions and cations are permeable during the early ATP response. Under conditions in which there was a large negative reversal potential for all cations, and a large positive reversal potential for all anions, the early ATP response reversed approximately 50 mV positive to the acetylcholine response.

8. The possibility that the early ATP response is due to a channel selective for size, but not charge, is discussed.

INTRODUCTION

When adenosine 5′-triphosphate (ATP) is applied to embryonic chick skeletal muscle, it has a profound excitatory effect (Hume & Honig, 1986; see also Kolb & Wakelam, 1983; Haggblad, Eriksson & Heilbronn, 1985). ATP is known to be co-localized with acetylcholine in some synaptic vesicles (Zimmermann, 1982; Castel, Gainer & Pellman, 1984), and in several cases has been shown to be released under conditions which stimulate transmitter release (Silinsky, 1975). For these reasons, we are interested in the possibility that ATP might act as a transmitter at developing neuromuscular junctions. Knowledge of the type of conductance changes activated by ATP is essential in assessing its role. Kolb & Wakelam (1983) reported the identification of a class of channels on embryonic chick skeletal muscle that seemed to be activated by ATP; however, our previous work (Hume & Honig, 1986) suggested that the macroscopic ATP response of chick muscle might not be generated by these channels. We found that the whole-cell responses showed profound long-term desensitization to prolonged application of ATP, while Kolb and Wakelam reported that single-channel events increased in frequency with time. To further address the nature of the conductance changes elicited by ATP, we decided to study the responses of myotubes to ATP under conditions in which most of the voltage-dependent channels would be blocked, so that we could observe the direct effects of ATP. The results reported here indicate that ATP elicits two currents, which can be distinguished on the basis of their time course and ionic selectivity. Surprisingly, the more rapidly activating current is characterized by increases in the conductance to both small cations and small anions. If this current is due to activation of a single class of channels, then the channels must be selective for size but not charge, since both large cations and anions appear to be much less permeable. Some of the results described here have been reported in preliminary form (Thomas & Hume, 1987).

METHODS

Cell culture

Standard chick embryo muscle cell cultures were prepared as described previously (Hume & Honig, 1986).

Intracellular recording

Intracellular recordings were made using conventional glass microelectrodes filled with 3 M-KCl as described previously (Hume & Honig, 1986). During initial experiments two independent electrodes were used to pass current and measure voltage. However, the high input resistance of muscle cells bathed in the blocking solution allowed the membrane potential to be varied between +20 and −100 mV by passing very small currents (< 1 nA). The resistance of electrodes was nearly constant when such small currents were passed; thus in our subsequent experiments we used a single microelectrode with a balanced bridge circuit both to record voltage and pass current (unless noted otherwise). The bridge circuit was balanced just prior to penetration of each cell.

Experiments were performed at room temperature (20–22 °C) unless specifically noted. In one set of experiments the temperature of the cells was maintained at 12 or 37 °C. Temperature was controlled by heating or cooling the metal plate on which the culture dish was mounted with a peltier device. The temperature in the dish was monitored with a miniature temperature probe and controlled with a feed-back device (all temperature control equipment made by Sensortek, Clifton, NJ, U.S.A.).

Extracellular solutions

Just prior to recording, each culture dish was washed at least three times over a period of 5 min with the appropriate external solution in order to replace the incubating media. Each wash exchanged about 3 ml. Recording was then performed over a period of up to several hours. All solutions contained 12·5 mM-HEPES (as buffer), and 30 μM-Phenol Red (as indicator) in order to maintain the pH between 7·3 and 7·4. The pH was adjusted with the appropriate hydroxide as indicated in Table 1. Our normal physiological solution contained (in mM): 132 NaCl; 5 KCl; 5·4 $CaCl_2$; 1·6 $MgSO_4$; 1·3 NaH_2PO_4; 6·3 glucose; 12·5 HEPES; and 4 NaOH.

TABLE 1. Composition of blocking solutions used (in mM)

	Name of blocking solution				
	Standard	Low Na^+	High K^+	Acetate	Glucuronate
NaCl	110	—	—	—	—
Sodium acetate	—	—	—	110	—
Sodium glucuronate	—	—	—	—	110
NaOH	5	5	5	5	1
KCl	4	4	—	—	—
Potassium acetate	—	—	114	4	—
KOH	—	—	—	—	4
Glucuronic acid	—	—	—	—	4
$MgCl_2$	1	1	1	1	1
TEA-Cl	20	120	20	20	20
Tetrodotoxin	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}
$CoCl_2$	4	4	4	4	4
HEPES	12·5	12·5	12·5	12·5	12·5
Phenol Red	0·03	0·03	0·03	0·03	0·03
Glucose	10	10	10	10	10

In some experiments most of the ions were replaced with an isosmotic amount of sucrose. The sucrose solution usually contained (in mM): 260 sucrose; 1 NaOH; 1 KOH; 1 $CoCl_2$; and 5 HEPES, but in a few experiments the $CoCl_2$ was omitted. Microelectrodes placed in solutions of low ionic strength can display substantial tip potentials, which may change when the electrode enters the normal ionic strength intracellular solution. For this reason, it is possible that the potentials we measured in cells bathed in this solution would differ from the true membrane potential by a constant offset. We cannot be certain that the true reversal potentials of responses measured in the sucrose solution were at exactly the values we report in Results. However, the purpose of these experiments was to determine whether the reversal potentials for ATP and ACh responses, which were quite similar in normal external solution, were also similar under these conditions. Both ATP and ACh responses were measured in each cell studied. In a single cell, a change in tip potential would offset the measured reversal potentials of both responses by the same amount, but any difference in the two reversal potentials could not be a consequence of a change in tip potential.

The compositions of all other solutions (the blocking solutions) are shown in Table 1.

Drug application

ATP and ACh were applied by pressure ejection from separate micropipettes as described previously (Hume & Honig, 1986). The drugs were always dissolved in the external solution in which the cells were bathed. Occasional cells hyperpolarized several millivolts in response to pressure application of external solutions alone. The time course of these hyperpolarizations was always very similar to the duration of the pressure pulse. We suspect that these small hyperpolarizations were artifacts induced by movement of the recording electrode in response to the pressure pulse, since they were absent when the puffer pipette was not aimed directly at the recording microelectrode.

Conductance measurements

In order to determine the effects of ATP on membrane conductance, the input conductance of myotubes was monitored before and during long applications of 10 μM-ATP. To measure input conductance, each cell was tested with 0·1 nA hyperpolarizing constant-current pulses 0·2 s in duration. Only cells in which the voltage response to the current injection reached a plateau within 0·2 s were used (so that the input conductance could be accurately measured at relatively short intervals). In addition, cells initially adjusted to -80 mV were only studied if their resting input conductance was less than 10 nS (to reduce the resting conductance relative to the ATP-specific conductance). Prior to ATP exposure the input conductance was determined for at least four different potentials spanning the range over which the ATP response was expected to occur.

During ATP application the membrane potential was changing as input conductance was being measured (see Fig. 6*B*). To analyse input conductance data, we first created an idealized trace that represented the shape that the trace would have had if the test current pulses had not been present. To construct each idealized trace the membrane potential just before the onset of each test pulse was measured and a smooth curve fitted to the points by eye. The idealized trace was then superimposed over the actual trace. For each test pulse the difference between the membrane poential at the end of the pulse and the membrane potential of the idealized trace was then measured. We calculated the total input conductance using Ohm's law, and then calculated the conductance specifically due to ATP by subtracting the non-ATP-dependent conductance appropriate to each potential from the total conductance. The potential was taken to be the value of the idealized trace at the time each test pulse ended. The non-ATP-dependent conductance at that potential was determined by linear interpolation from the data collected in the absence of ATP. The ATP-specific conductance at 0·2 s after the beginning of ATP application was assigned the value of 0·0 nS in order to nullify any artifacts associated with the pressure pulse. In any case, the average conductance at 0·2 s was almost identical to that at 0·2 s before the onset of ATP application.

RESULTS

ATP had a potent excitatory action on myotubes bathed in a solution that approximated normal extracellular ionic conditions (normal solution). In normal solution, brief application (0·5 s) of 50 μM-ATP caused a majority of myotubes (77 %, $n = 2000$) to contract. Intracellular recordings from myotubes bathed in normal solution (Fig. 1*A*) revealed that ATP elicited a rapid depolarizing spike followed by a long-lasting (tens of seconds) after-depolarization. The peak of the action potential was typically near 0 mV, while the after-depolarization was typically near -20 mV. Application of normal solution without ATP from a second pipette was usually without effect (Fig. 1*A*). When brief depolarizing current pulses were given to muscle cells (Fig. 1*B*), a spike and after-depolarization were also elicited. This suggested that the true time course of the ATP-evoked response was probably obscured by the depolarization caused by the activation of voltage-dependent currents. The currents that give rise to active responses in chick myotubes have been studied extensively (Kano, Shimada & Ishikawa, 1972; Kano & Shimada, 1973; Fukuda, 1974; Fukuda, Fischbach & Smith, 1976). These cells are known to possess voltage-dependent Na^+ and Ca^{2+} conductances which are responsible for the spike and a Cl^- conductance which is responsible for the after-depolarization. In addition these cells possess K^+ channels which are activated during depolarization. In order to block the major voltage-dependent responses in these cells, a Ca^{2+}-free external solution containing tetrodotoxin (TTX), tetraethylammonium (TEA) and Co^{2+} was used (blocking solution). In this solution, cells had resting potentials that varied from -60 to -10 mV (average near -25 mV). As was true for cells bathed in the normal solution, resting potentials became more negative with increasing age and fibre size. Muscle

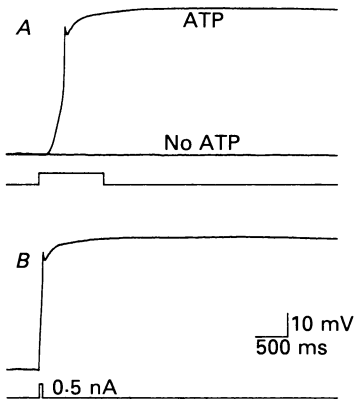

Fig. 1. ATP excites chick muscle in normal solution. *A*, pressure application of 10 μM-ATP dissolved in normal solution evoked a large depolarization. Pressure application of normal solution without ATP caused no potential change. The lower trace indicates the duration of the pressure pulses. Both responses were from the same cell. The initial membrane potential was −70 mV. *B*, the response of the same cell as in *A* to a 0·5 nA depolarizing current injection passed through the recording electrode. Depolarization alone can elicit a spike and long-lasting after-depolarization. The initial potential was again −70 mV. The lower trace indicates the duration of the current pulse.

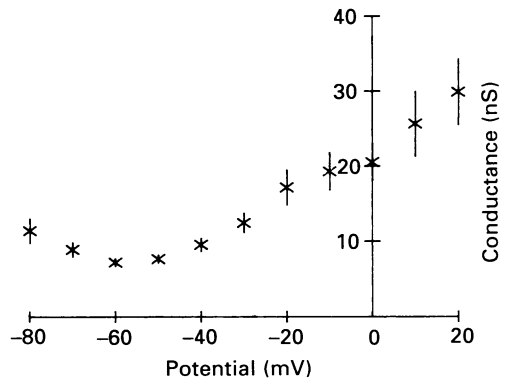

Fig. 2. Average input conductance as a function of membrane potential in the standard blocking solution. The blocking solution eliminated much of the non-linearity of the current–voltage curve. The value at each potential is the average of measurements taken from the same eight cells. Each cell was impaled with two microelectrodes, one for passing current, the other for recording membrane potential. A steady current was passed to set the initial potential, then a 0·1 nA hyperpolarizing current step was given, and the resulting potential deflection was measured. Conductance was calculated using Ohm's law. Error bars represent the standard error of the mean (S.E.M.).

cells with shallow initial resting potentials could often be brought to a new stable resting potential below −25 mV by passing a small (1 nA) hyperpolarizing current through the recording electrode for approximately 1 min. During this time the input resistance increased dramatically. After the membrane potential and input resistance had stabilized, the steady-state input conductance in the blocking solution showed only gradual changes over a broad range of membrane potentials (Fig. 2).

Furthermore, rapid changes in membrane potential usually failed to reveal any obvious voltage-dependent responses. Large depolarizing current pulses from negative membrane potentials did not result in either a potential spike or after-depolarization (Fig. 3*A*). Occasionally, a cell's input resistance dropped significantly in response to the first depolarizing current pulse the cell received. However, after several pulses the input resistance usually showed much less of a non-linear component.

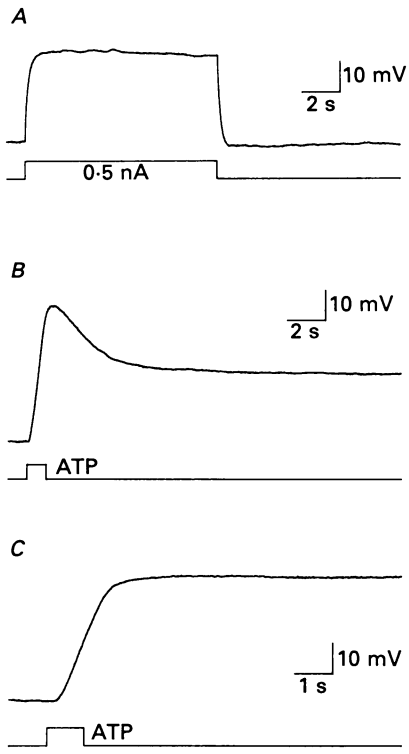

Fig. 3. The responses to depolarizing current injection and ATP in the standard blocking solution. Voltage-dependent active responses were eliminated in the blocking solution but ATP responses were still present. *A*, the response to a 10 s, 0·5 nA injection of depolarizing current through the recording electrode. *B*, the response to a 1 s application of 10 μM-ATP from the same cell as in *A*. Most cells showed biphasic responses to ATP. *C*, the response to a 1 s application of 10 μM-ATP from another cell. In occasional cells there was no indication of a second phase. For both *A* and *B* the potential was initially at -90 mV, while for *C* it was initially at -70 mV.

In the absence of the major voltage-dependent currents the actual response to ATP could be observed. When the membrane potential was initially adjusted to a value more negative than -20 mV (by passing a constant current through the recording electrode) the initial response to a brief application of ATP was a relatively rapid depolarization which peaked in 1–5 s. Most cells then partially repolarized (that is, became more negative with respect to peak depolarization) in 3–10 s. An example is shown in Fig. 3*B*. Depolarizing current pulses that brought cells to the same

potential as that induced by ATP never elicited a repolarizing response (Fig. 3*A*). Thus, the repolarizing phase of the ATP response was not a result of membrane depolarization alone. Full return to the initial membrane potential often took a very long time (> 60 s).

The dual effect of ATP

There were several possible explanations for the repolarizing phase of the ATP response. They included diffusion of ATP away from the fibre at the termination of ATP application, desensitization of the ATP response and conductance changes distinct from those affected during the initial phase of the response. To test these possibilities, the responses of fibres to long applications of ATP over a wide range of membrane potentials (−80 to +10 mV) were recorded.

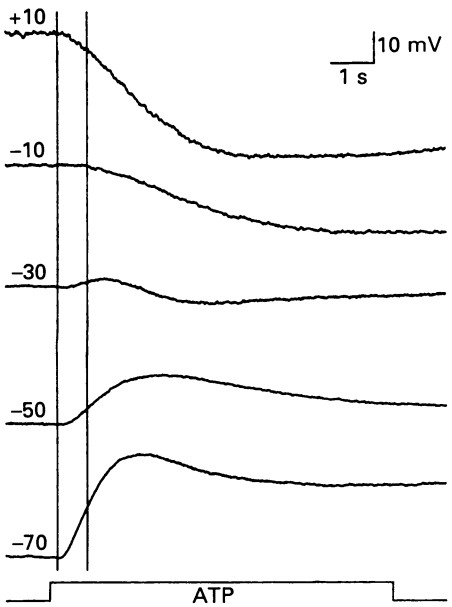

Fig. 4. Responses to long applications of ATP at different initial membrane potentials. These five traces show the responses of five different cells of the same muscle culture to application of 10 μM-ATP. The potential of each cell was adjusted to the level indicated above each trace (mV), and ATP was applied for 8 s. The two vertical lines highlight the early phase of the ATP response. The cells were bathed in the standard blocking solution.

Several noteworthy observations are illustrated in Fig. 4. First, the repolarizing phase of the response to ATP began within a few seconds, even though ATP continued to be present. Thus the repolarizing phase was not due to a lack of ATP. Second, at −30 mV the second (repolarizing) phase of the response was often seen to be hyperpolarizing with respect to the initial membrane potential. This indicated that the second phase was not merely a result of desensitization of the first (depolarizing) phase of the response, since complete desensitization would have only

returned the membrane to its initial potential. Third, within the first second of ATP application the response reversed near −10 mV, while at times after 1 s, the apparent reversal potential of the response was more negative. Finally, the latency between ATP application and the onset of a response was significantly longer at −10 mV than at the other potentials shown. The latency to the onset of the response to ATP for a population of cells is illustrated in Fig. 5. With our usual method of ATP application, the measured latency at most potentials was about 400 ms (but see below), but the average latency was prolonged to over 1 s at −20 and −10 mV.

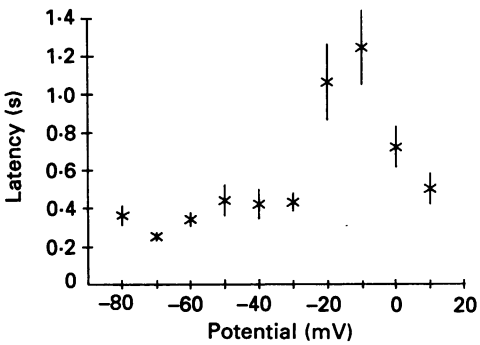

Fig. 5. Latency to the onset of the response to ATP as a function of membrane potential. The latency to the onset of ATP responses is greatly prolonged at −10 and −20 mV. Latency was measured as the time from the beginning of the command to pressure-eject ATP to the beginning of the response. Pipettes were positioned approximately 50 μM from the cells. As described in the text, dead time in the delivery system accounts for most of the measured latency at potentials other than −10 and −20 mV. Myotubes were bathed in the standard blocking solution, and were from the same muscle culture. Each point is the average of ten cells, and error bars represent the S.E.M.

Taken together, these results suggest that the response to ATP has two distinct phases: a more rapidly activating component which has a reversal potential near −10 mV, and a more slowly activating component that reverses at a significantly more negative potential.

Under our standard recording conditions the measured latency until the onset of the early phase of the ATP response was about 400 ms. However, some part of this latency represented the lag time of our drug delivery system. We usually kept our drug application pipettes relatively far from the myotubes, in order to prevent desensitization due to possible leakage of ATP out of the pipette tip. To get a better estimate of the actual latency to the onset of ATP responses, we compared the responses to a high-K^+ solution applied from one pipette with the responses to ATP applied from a second pipette at a similar distance from the muscle cell. The idea behind this experiment was that the potential changes due to application of high K^+ and application of ATP are similar, but because the conductance to K^+ is already high, K^+ could begin to depolarize cells as soon as it reached them, while ATP could not begin to depolarize cells until channels opened. Thus the difference between the ATP- and K^+-evoked latencies to depolarization provides a rough estimate of the

actual latency of the early ATP response. On average, ATP responses had a latency that was about 20 ms (S.E.M. = 3) greater than K^+ responses. Thus the latency to activation of the early component of the ATP response is probably no more than 20 ms.

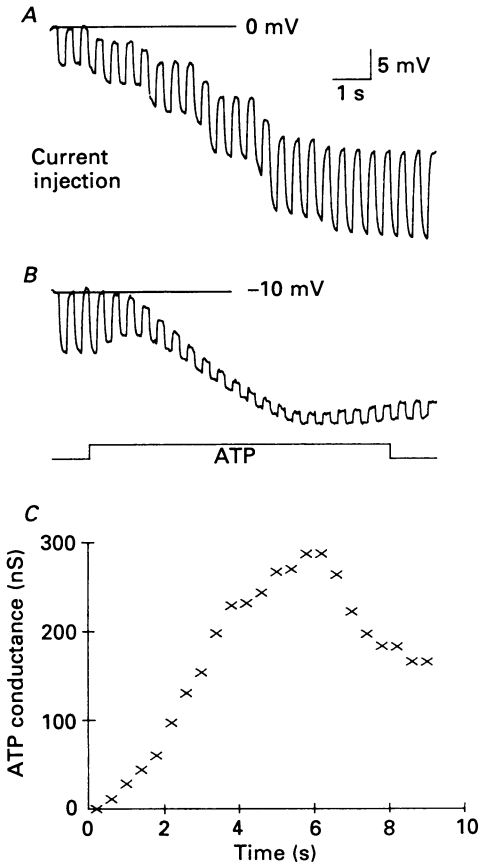

Fig. 6. ATP-specific conductance for one cell initially at −10 mV. *A*, this cell was passively hyperpolarized during a train of 0·5 nA hyperpolarizing constant-current pulses in order to determine conductance as a function of voltage (see Methods). *B*, the cell was then exposed to an 8 s application of 10 μM-ATP during a second train of current pulses. *C*, ATP-specific conductance (calculated as described in Methods) is plotted as a function of time. Each point of the graph corresponds to the pulse directly above it in *B*. The initial input conductance of the cell at −10 mV was 50 nS. The cell was bathed in the standard blocking solution.

ATP-specific conductance changes

To better understand the mechanism by which ATP acts, conductance measurements were made during long applications of ATP. In the first set of experiments a constant current was passed so that each cell was initially adjusted to a potential near −10 mV. As cells were passively hyperpolarized from −10 to −30 mV, the input conductance in the absence of ATP gradually decreased, as shown by the larger potential changes in response to hyperpolarizing constant-current injections (Fig. 6*A*, see also Fig. 2). In contrast, the input conductance

increased dramatically during the application of ATP (Fig. 6*B*). The conductance change due specifically to ATP (Fig. 6*C*) was derived from these two records (see Methods). In cells initially adjusted to −10 mV, there was little change in membrane potential during the first second of ATP application, despite a clear increase in conductance. This is consistent with the idea that ATP activates an early current which reverses near −10 mV. Over the next few seconds, as the cell hyperpolarized in response to ATP, the input conductance continued to increase, and finally peaked as cells reached maximum hyperpolarization.

Conductance measurements were also made on eleven cells adjusted to an initial membrane potential of −80 mV. This allowed us to determine whether the time course of the conductance increase depended on membrane potential. Similar to the results found for cells with an initial potential of −10 mV, the average ATP-specific conductance of cells initially at −80 mV increased slowly at first, and then more rapidly (Fig. 7*B*). Conductance increased at nearly a constant rate (1·3 nS/s) between 0·2 and 1·8 s. After 1·8 s, the conductance continued to increase, but at an even greater rate, reaching a maximum of 2·8 nS/s. By 9 s, the average conductance of these cells was 2·3 times the original conductance, and had almost reached a plateau.

Significantly, the conductance was still increasing at the average time (dashed line, Fig. 7*B*) that the response reached maximum depolarization (at an average membrane potential of −36·7 mV). Since this potential was far from the reversal potential for the early response (near −10 mV, Figs 4 and 6*B*), a second, opposing conductance change must also have been present at the time of peak depolarization. The presence of two separate conductance changes also was suggested by the shift in the rate of conductance increase around 1·8 s both for cells initially at −10 and −80 mV. This time point corresponded relatively well with the 1·24 s latency to the beginning of the ATP response for cells near the reversal potential of the early response (−10 mV, Fig. 5).

A simple interpretation of the conductance measurements is that the initial rate of conductance increase represents the activation of the set of channels responsible for the early depolarizing response, while the greater rate of conductance increase that follows represents the activation of the second component as well. However, an alternative is that the initial conductance change represents the time course of activation at the site nearest the pipette delivering ATP, and that the later increase in the rate of conductance change represents the additional conductance recruited as ATP spreads to more distant regions of the muscle cell. Two observations argue against the latter idea being responsible for the pattern of ATP conductance increase. First, short (1 s) applications of ATP elicited responses similar in waveform to long applications (Fig. 3*B*). Second, long applications of acetylcholine (ACh) to cells did not give a biphasic increase in the conductance, but rather gave a very rapid, monophasic conductance increase (Fig. 7*B*). ACh receptors are known to be distributed diffusely over the surface of muscle cells (Sytkowski, Vogel & Nirenberg, 1973). If the time course of the ATP conductance change represented delayed activation of distant receptors, then ACh should have given a similar response. Thus we conclude that both the early depolarizing response and the late hyperpolarizing response are due to conductance increases.

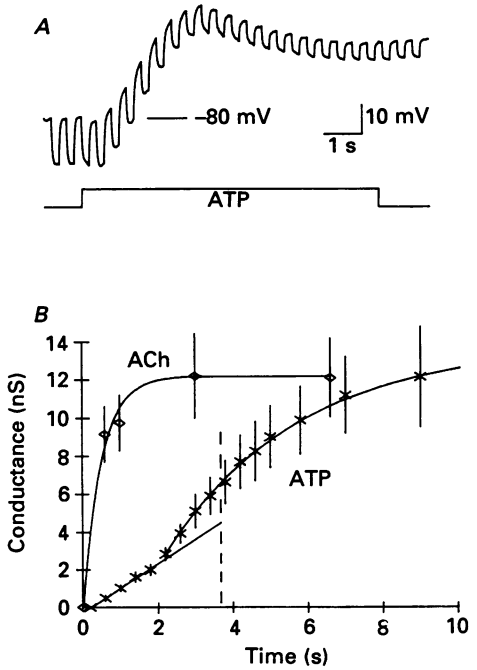

Fig. 7. Average ATP- and ACh-specific conductance changes for cells initially at −80 mV. *A*, record of the conductance measurements from one cell exposed to ATP. *B*, average conductance response from eleven cells. To observe ACh responses the TEA in the standard blocking solution was replaced with Na^+. Error bars represent the S.E.M. The vertical dashed line was drawn at the average time that the membrane potential reached its peak. The extension of the initial slope of the ATP response to this line was drawn for the following reasons. If the capacitance of the membrane is ignored, due to the slow time course of the response, then the membrane potential depends on three parallel paths representing the leakage conductance and the two conductance pathways due to ATP action. At peak depolarization, $dV/dt = 0$, and at the time when $dV/dt = 0$, the following equation can be derived from the equivalent circuit:

$$(dG_{early}/dt)/(dG_{late}/dt) = (V_m - E_{late})/(E_{early} - V_m),$$

where G refers to conductance, E to equilibrium potential, 'early' to the first component of the ATP response, 'late' to the second component of the ATP response, and V_m to the potential at peak depolarization. For the eleven cells measured from an initial potential of −80 mV, the peak occurred at an average time of 3·7 s (S.E.M. = 0·4) and at an average potential of −36·7 mV (S.E.M. = 2·6). Experimentally derived values of $E_{late} = -80$ mV and $E_{early} = -17$ mV yield $(dG_{early}/dt)/(dG_{late}/dt) = 2{\cdot}20$. Since the total rate of conductance increase due to ATP $(dG_{early}/dt + dG_{late}/dt)$ was 2·0 nS/s at 3·7 s, $dG_{early}/dt =$ 1·37 nS/s, very close to the initial slope of 1·3 nS/s. Varying any value by its S.E.M., or E_{late} by 10 mV, resulted in less than a 10% change in this value. Similar results were obtained when analysing single-cell responses. By these estimates then, the rate of the conductance increase for the early response probably remained almost constant during the first 4 s.

Variability in the second phase of the ATP response

The biphasic responses described in the rest of this paper were found in most cells. However, in some cells the second component appeared to be absent. When these cells were set to the reversal potential of the early response, no late hyperpolarization

was seen, and when ATP was applied to cells at resting potential it produced a rapid depolarization to a maintained peak (Fig. 3*C*). These observations indicate that desensitization of the early response may take many seconds. Thus for cells that show biphasic responses, it is likely that the repolarization represents almost entirely the effect of the second phase. Among cells with biphasic responses the relative amplitude of the two phases varied substantially from cell to cell. Variability in the duration of the second phase of ATP action was also noticeable between cells. For instance, close inspection of Figs 6*C* and 7*B* shows that the conductance of the cell

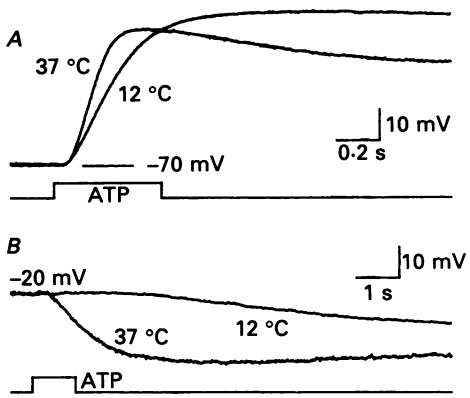

Fig. 8. Temperature sensitivity of the two responses to ATP. *A*, to explore the temperature sensitivity of the early response, cells were set to an initial potential of −70 mV. Responses from cells of similar input resistance (120 and 105 MΩ respectively) at 12 and 37 °C to 0·5 s applications of 50 μM-ATP are illustrated. Cells were bathed in the standard blocking solution. *B*, to explore the temperature sensitivity of the late response cells were set to an initial potential of −20 mV. Responses from cells of similar input resistance (21 and 24 MΩ respectively) at 12 and 37 °C to 1 s applications of 10 μM-ATP are illustrated. Cells were bathed in the acetate-blocking solution to enhance the late response.

initially at −10 mV began to fall from its peak prior to 9 s, while the average conductance for the sample of cells initially at −80 mV was still increasing at that time. This is likely to represent cell-to-cell variability, rather than an influence of voltage on the time course of the ATP response, since some cells initially at −80 mV also achieved their peak conductance prior to 9 s. The overall similarity in the pattern of the conductance increases from such different initial potentials indicates that there is probably no pronounced voltage dependence of the conductance changes elicited by ATP.

Temperature dependence of the ATP responses

Since the time to activation of the early response to ATP was at most 20 ms, it seemed possible that opening of channels was a direct effect of activation of the ATP receptor. Not only should a directly activated response have a short latency, but the overall time course and size should be relatively insensitive to temperature changes. To test this idea, measurements estimating the rate of activation and the magnitude of the two ATP responses at 12 and 37 °C were made (Fig. 8). The magnitude of the

early response was nearly the same at these two temperatures, and the time to half-maximal activation increased less than twofold when the temperature was dropped from 37 to 12 °C (Table 2). In contrast, the magnitude of the late response at 12 °C was approximately one-third that at 37 °C, and the time to half-maximal activation was almost five times longer when the temperature was dropped from 37 to 12 °C. These results support the idea of direct activation of the early response by ATP, and also suggest that some intermediate biochemical process may link receptor activation to the late response.

TABLE 2. Effect of temperature on the two components of the ATP response

	−70 mV	−20 mV
Temperature (°C)	Time to half-maximal response (s)	
37	0·116 ± 0·004 (8)	0·9 ± 0·08 (10)
12	0·205 ± 0·018 (15)	4·14 ± 0·43 (10)
	Estimated peak current (pA)	
37	295 ± 25 (8)	910 ± 116 (10)
12	261 ± 26 (15)	292 ± 42 (10)

Cells were initially adjusted to −70 mV to assess the early response, or to −20 mV (near the reversal potential for the early response) to assess the late response. To estimate the rate of activation for each response the time from the beginning of the ATP response to the time at which the response reached half of its maximal potential change was measured. An estimation for the peak current of the response was made by dividing the peak depolarization from −70 mV or the peak hyperpolarization from −20 mV by the initial input resistance. Conditions were those described in Fig. 8. Values are ± the S.E.M., and the numbers in parentheses are the cell sample size.

Ionic dependence of the ATP responses

The relative permeabilities of ions through a set of channels can be estimated by substituting one ion in the external solution with another, and then measuring the shift in reversal potential (Hille, 1984). In our experiments, the interpretation of ion substitutions is potentially more complex because two different currents are being activated. We made the simplifying assumption that at 1 s so little of the second component had yet been activated that the response at this time was a result of only the first component. This was based on the observation that at the reversal potential for the first component, the latency to hyperpolarization was greater than 1 s, and that the inflexion point in the conductance increase also occurred later than 1 s. It was not possible to study the second component in isolation, so we could not directly estimate its reversal potential, but by studying cells in which the driving force for the early component was depolarizing relative to resting potential it was possible to make minimum estimates of the reversal potential for the late component (see below).

In the standard blocking solution, the estimated reversal potential of the early response was −17 mV (Fig. 9*A*). A reversal potential of this value could be due to a non-specific cation channel or to an anion-selective channel. In order to distinguish between these possibilities, most of the Na^+ or Cl^- in the blocking solution was replaced by another ion.

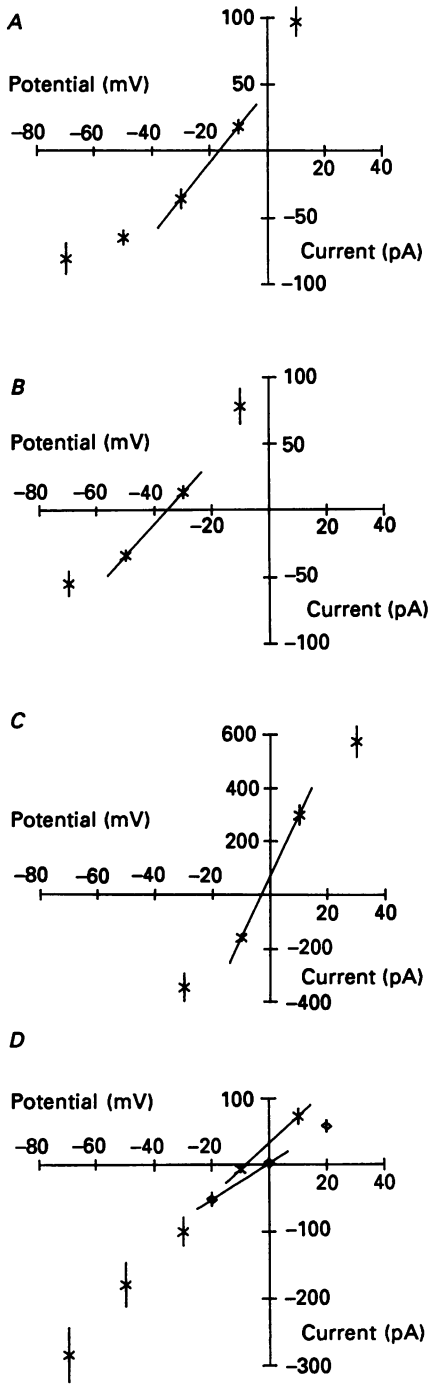

Fig. 9. For legend see facing page.

Sodium dependence

When 96% of the Na^+ in the blocking solution was replaced with the much larger cation TEA (low-Na^+ solution), the reversal potential of the early response was estimated to be −36 mV (Fig. 9*B*). This shift of the reversal potential suggested that Na^+ is permeable through the early ATP-activated channel and that TEA is much less permeable than Na^+. When the potential of cells bathed in low-Na^+ solution was adjusted so that they were near the reversal potential for the early response, these cells still showed substantial late hyperpolarizations (Fig. 10*A*). The most negative potential reached during the ATP response provided a minimum estimate for the reversal potential of the late response. This will be an underestimate, since the cell will not reach the reversal potential of the late response due to the other depolarizing conductances that are also active. By these criteria, the reversal potential of the late response appeared to be below −70 mV. A qualitative comparison of the amplitude of the hyperpolarizing responses suggested that there was little difference between the standard and low-Na^+ blocking solutions. Thus, neither Na^+ nor TEA appeared to be permeable during the late response.

Potassium dependence

In a second blocking solution, 96% of the Na^+ was replaced with K^+ (high-K^+ solution). In these experiments acetate was used as the major anion because of its ability to augment the late response (see following section). The average resting potential in this high-K^+ solution was approximately −5 mV, and the cells had low input resistances (ranging from 10 to 40 MΩ). In this solution the early response reversed at −3 mV (Fig. 9*C*). In the control acetate blocking solution (high Na^+ instead of K^+), the early response reversed at −8 mV (Fig. 9*D*), so the reversal of the early response at −3 mV for cells in the high-K^+ (also acetate) solution indicated that replacing 96% of the Na^+ with K^+ had caused a shift of +5 mV in the reversal

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Fig. 9. Current–voltage relationships for the early ATP response in altered external blocking solution. Currents were estimated by dividing the change in membrane potential at 0·7 or 1 s after the onset of ATP application by the initial input resistance. Each point represents the average current from ten cells at that potential, and error bars represent the S.E.M. A line was drawn through the two points closest to zero current to estimate the reversal potential. The lines used in the interpolation often did not pass through the other points; in particular, values at negative potentials were often smaller than expected. At least part of this apparent rectification is likely to be the result of the residual voltage dependence of the passive current–voltage relationship in these cells, and the method used for normalization. Over the range of −50 to 0 mV the input conductance gradually increases. Since the normalization calculation uses the initial input resistance value, normalization would be expected to underestimate the amplitude of the currents at more negative potentials. Some of the rectification observed might also be due to the properties of individual ATP-activated channels. *A*, responses measured in the standard blocking solution at 1 s. *B*, responses measured in the low-Na^+ blocking solution (96% of Na^+ was replaced by TEA) at 1 s. *C*, responses measured in the high-K^+ blocking solution (96% of Na^+ was replaced by K^+, and 79% of Cl^- was replaced by acetate) at 1 s. *D*, responses measured in the low-Cl^- blocking solutions (79% of Cl^- was replaced by either acetate, ×, or glucuronate, ◇) at 0·7 s.

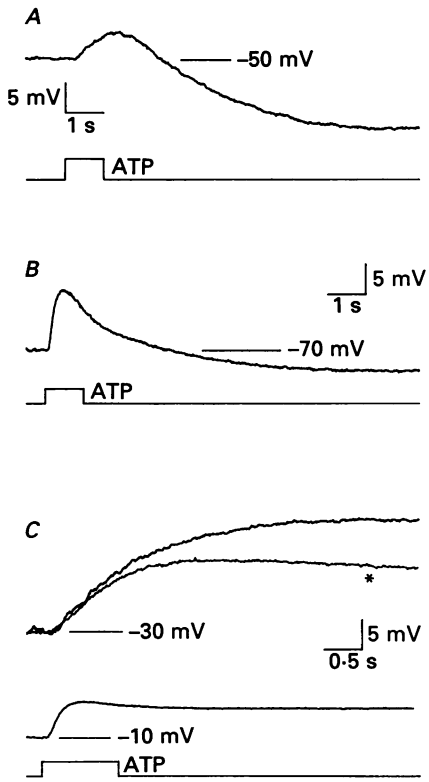

Fig. 10. Potassium dependence of the second phase of the ATP response. Changing external Na^+ or Cl^- concentration did not noticeably alter the reversal potential of the late response, while raising extracellular K^+ had a dramatic effect. *A*, in this recording from a cell bathed in low-Na^+ solution the potential of the cell was initially set to −50 mV. The late phase of the response hyperpolarized the cell to −62 mV, so its reversal potential must be negative to this level. *B*, in this recording from a cell in acetate solution (low Cl^-) the potential of the cell was initially set to −70 mV. The late phase of the ATP response hyperpolarized this cell to −74 mV, so its reversal potential must be negative to this level. *C*, these recordings were made from cells bathed in high-K^+ solution. In the top two records the potential of each cell was initially set to −30 mV. The response marked with the asterisk (*) came from a cell in a culture that showed no biphasic responses when studied in standard blocking solution. In this cell the ATP response rapidly reached a plateau. The response from the other cell came from a culture that showed robust biphasic responses in standard blocking solution. The cell showed a rapid initial phase of depolarization, and then a slower phase of depolarization. The difference between these two traces is taken to indicate the effect of the second phase of the ATP response. Thus in this solution the reversal potential of the late phase must be positive to the final value of −11 mV. In the bottom record the potential was initially set to −10 mV. ATP evoked an initial depolarization to −4 mV, and then a small repolarization to −5 mV. Thus the reversal potential for the late response is likely to be more negative than −4 mV.

potential. This shift implies that the early response is slightly more permeable to K^+ than to Na^+.

Several observations suggest that in the high-K^+ solution the reversal potential of the late response was similar to that of the early response. In high-K^+ solution the

typical response to ATP consisted of a rapid potential change over the first few seconds, and then a slower change in the same direction over the next few seconds (Fig. 10*C*). This is the result expected for activation of two conductances with different time courses but similar reversal potentials. An alternative, that this pattern of potential changes represented only the first phase, and that the late response was absent because it was blocked by the high external K^+ concentration, seems unlikely for two reasons. First, in a culture with greatly diminished late responses in the standard blocking solution (a result of the culture-to-culture variability mentioned earlier), the slower polarizations were not present in the high-K^+ solution (Fig. 10*C*, trace with asterisk). Second, some cells initially adjusted to -10 mV rapidly depolarized in response to ATP, stayed briefly at a plateau and then repolarized slightly to a second plateau potential (Fig. 10*C*, bottom trace). This biphasic response suggested that the late response was present in these cells and had a reversal potential that was similar, but not identical, to that of the early response. We conclude from these observations that the late response, which normally reversed more negative than -70 mV, reversed between -10 and -5 mV in the high-K^+ solution. This change of 60 mV in the reversal potential indicates that the channels responsible for the late response are much more permeable to K^+ than to Na^+.

Chloride dependence

Although the ability of changes in external Na^+ and K^+ to shift the reversal potential of the early response suggested that the channel opened by ATP was a cation channel, anion permeability was also tested. In the first series of experiments 79% of the Cl^- was replaced with the larger anion acetate. This change resulted in a reversal potential of -8 mV for the early ATP response (Fig. 9*D*, ×), a shift of $+9$ mV from the reversal in the standard blocking solution. This shift was in the direction expected if Cl^- was permeant through the channel, but was much smaller than expected if the channel was permeable exclusively to Cl^-. One explanation for this small shift could be that acetate, the ion used to substitute for Cl^-, might also permeate the channel. To test this possibility, the much larger anion glucuronate was used to replace 79% of the Cl^- (Fig. 9*D*, ◇). In this solution the reversal potential of the early response was -1 mV. The larger shift in reversal potential with glucuronate as compared to acetate suggests that both Cl^- and acetate are significantly permeable during the early phase of the ATP response. However, even with this very large anion present, the change in reversal potential was not nearly as large as would have been expected for a pure Cl^- conductance.

Taken together with the earlier data showing that alterations in cation concentration can also change the reversal potential, these data are consistent with the idea that the channels opened during the early phase of the response to ATP allow both small cations and small anions to permeate the membrane. However, an alternative explanation is that changes in extracellular Cl^- may have resulted in a redistribution of intracellular cations (for instance an efflux of potassium) and thus altered the reversal potential of a cation-selective response. Because of this concern, a second type of experiment was performed to test Cl^- permeability during the early ATP response. Muscle cells were bathed with an extracellular solution in which most of the permeant ions had been replaced by sucrose (sucrose solution, see Methods).

In such a solution the reversal potential for Na^+ and K^+ should be quite negative, and the reversal potential for Cl^- should be quite positive. Because ACh receptor channels are known to be highly selective for cations over anions (Adams, Dwyer & Hille, 1980), each cell was tested with ACh and then ATP (Fig. 11). If ATP activated a cation channel, then ACh and ATP responses should have similar reversal potentials. The response to ACh typically reversed between −70 and −80 mV,

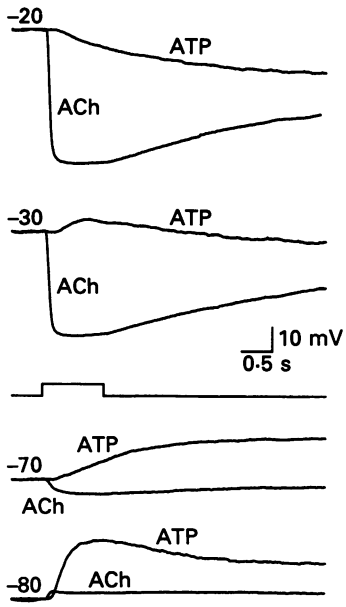

Fig. 11. ATP and ACh responses of muscle cells bathed in a low ionic strength external sucrose solution. In this solution it would be expected that the reversal potential for a cation conductance would be very negative, while the reversal potential for an anion conductance would be very positive. One second applications of first 10 μM-ACh and then 50 μM-ATP were given to the same cell for each potential tested (mV). The cell was allowed to return to the initial potential prior to application of ATP. More than thirty cells were tested in the solution containing 1 mM-$CoCl_2$ over the potential range of 0 to −100 mV in order to determine the approximate reversal potential for each response. Responses from four representative cells demonstrate the results. The sucrose solution contained 1 mM-cobalt because divalent cations greatly improved the quality of the impalements. Cobalt was chosen because other experiments (not shown) indicate that it does not permeate the early conductance activated by ATP. Recordings made from a few cells in which the sucrose solution contained no divalent ions confirmed this result, since the reversal potential of the ATP response estimated from these cells was not different from that measured when cobalt was present.

consistent with that expected for a cation-selective process. On the other hand, ATP evoked an early response that reversed between −20 and −30 mV, and a late response that reversed significantly more negative than −60 mV (note the record at −80 mV, Fig. 11). Since the reversal potential for both phases of the ATP response was negative we conclude that cations are significantly more permeable than anions during each phase. However, since the initial component reversed at a much less

negative value than the potential at which the second component or the ACh response reversed, then there must also have been a substantial increase in Cl^- permeability during the early phase of the ATP response.

In regard to the second phase of ATP action, repolarizations and hyperpolarizations occurred at similar potentials in the standard and low-Cl^- blocking solutions (Fig. 10*B*). This suggested that the reversal potential for the late response was not changed by substitution for Cl^-. In fact, the acetate- and glucuronate-substituted solutions appeared to increase the magnitude of the late response at most potentials. The reason for this potentiation is unclear. The increase in the amplitude of the late response was in the opposite direction from that expected if Cl^- were permeable through the channels responsible for the late response. Given the large shift in the reversal potential of the late response produced by changes in K^+ concentration, and the absence of obvious effects of Na^+ and Cl^- substitution, it seems likely that the late response is highly selective for K^+.

In summary, these results imply that ATP first activates a channel or set of channels that allows Na^+, K^+ and Cl^- ions to cross the membrane, and soon thereafter activates channels selective for K^+ ions.

DISCUSSION

Cultured chick myotubes, bathed in an extracellular solution that approximates interstitial fluid, depolarized from resting potential and contracted upon application of micromolar concentrations of ATP. When muscle cells were bathed with a Ca^{2+}-free solution containing blockers of several types of voltage-dependent channels, the myotubes no longer contracted, but they still depolarized from resting potential in response to ATP. Furthermore, when the blockers were present, myotubes responded nearly passively to constant-current injections over a wide range of membrane potential, indicating that the voltage-dependent channels were indeed blocked. In most cases the ATP-evoked depolarizations from resting potential were followed within several seconds by partial repolarizations. Experiments measuring the voltage dependence and temperature dependence of the response to ATP, and experiments measuring conductance during the application of ATP, all suggested that two separate currents were activated by ATP. Both currents appeared to be due to increases in conductance. In the standard blocking solution the more rapidly activating current had a reversal potential near -17 mV, while the more slowly activating current reversed more negative than -70 mV. Ion substitution experiments confirmed that two different currents were present.

The ion substitution experiments showed that during the early phase of ATP action all three of the predominant physiological ions, Na^+, K^+ and Cl^-, appeared to be significantly permeable. Because we found this result surprising, we performed an additional experiment. By replacing virtually all of the permeant ions with isotonic sucrose we created conditions under which the reversal potential for cations would be very negative, and the reversal potential for anions would be very positive. In this solution the observed reversal potential for the early phase of the ATP response was between -20 and -30 mV, a value much too positive for a pure cation conductance (as demonstrated by the ACh response), but consistent with a simultaneous increase

in the permeability to cations and anions. However, the fact that the reversal potential is negative suggests that the permeability to cations is greater than the permeability to anions. One can calculate the relative permeability to Na^+, K^+ and Cl^- using the Goldman equation. Because we assumed values for internal ion concentrations, and also assumed that they do not change during ion substitution experiments, such calculations are only approximate. The values we used for internal ion concentrations were 95, 15 and 80 mM for K^+, Na^+ and Cl^- respectively. The relatively high value used for intracellular Cl^- was based on the peak potential for the long-duration, Cl^--dependent spike (−15 mV). Calculations using these concentrations yielded values for pNa^+/pK^+ and pCl^-/pK^+ in the range of 0·3–0·7. However, no pair of ratios could predict the results of all of the ion substitution experiments. For instance, if one solved for the ratios using the change in reversal potential going from the standard blocking solution to the low-Na^+ blocking solution, one obtained $pNa^+/pK^+ = 0{\cdot}39$ and $pCl^-/pK^+ = 0{\cdot}38$. Substituting these numbers back into the Goldman equation produced reversal potentials with anion substitutions and with high K^+ that were close but not identical to the experimentally observed values. It would not be surprising if this quantitative discrepancy was a consequence of our having to estimate reversal potentials by interpolation from single responses of a population of cells, or a consequence of the internal ion concentrations changing somewhat depending on the external solution used.

The observation that permeability to both cations and anions increased during the early ATP response is intriguing. In current models of channel selectivity, size discrimination is thought to result from a physical constriction within the pore, while charge selectivity is thought to result from an excess of fixed charges of one sign within the pore (Hille, 1984). If the early response is the result of activation of a single class of channels, then one may speculate that the pore is similar in size to the ACh receptor, since it does not allow TEA or glucuronate to cross the membrane, but that the charge is much more evenly balanced within the channel so that both positive and negative ions can permeate the membrane. In regard to non-specific channels, it may be relevant that ATP can apparently open a permeability pathway in rat mast cells that does not discriminate between positively and negatively charged molecules (Cockcroft & Gomperts, 1979). However, the mast cell channel is clearly very different from that found in chick muscle in that it allows very large molecules (for instance sugar phosphates and nucleotides) to cross the membrane. An alternative mechanism to a channel that does not discriminate by charge would be activation of separate cation- and anion-selective channels with very similar time courses. A third, less likely mechanism for the early ATP response would be activation of an electrogenic ion transporter. Such a transporter would have to be symmetrical in its properties in order to give the apparent reversal potential and conductance changes that we have measured. In addition to distinguishing between these possibilities, it will also be of interest to determine the permeability of divalent cations during the early response.

The ionic mechanism responsible for the late response to ATP appears more straightforward. Even though reversal potentials could not be measured directly, changes in external K^+ concentration clearly affected the response, while large changes in extracellular Na^+ or Cl^- had no obvious effect. The simple conclusion is that the late response is due to the activation of a K^+-selective channel.

The difference in the rate of activation between the two phases of the ATP response suggests the possibility of different mechanisms of activation. We have shown that the latency to the onset of the early component is less than 20 ms. Such a short latency seems most consistent with direct activation by ATP of the ion channel(s) responsible for the early response. In contrast, the latency of approximately 1 s for the late response suggests that some biochemical process may be involved. The much greater temperature sensitivity of the second phase of the ATP response is also compatible with this idea. One reasonable possibility is that some second messenger system is activated by ATP and leads to the late response. Interestingly, Haggblad & Heilbronn (1987) have demonstrated an increase in the turnover of inositol phosphates subsequent to ATP exposure.

There are striking similarities between the ATP response that we have studied in chick skeletal muscle and ATP responses in other types of muscle. Friel & Bean (1988) examined currents elicited by ATP in dissociated bull-frog atrial cells. They observed an early, desensitizing response that reversed near −10 mV, and a late response that reversed at −85 mV. There was little selectivity among monovalent cations during the early response; anion permeability was not directly tested. Benham, Bolton, Byrne & Large (1987) and Benham & Tsien (1987) observed a transient, desensitizing response to ATP in dispersed rabbit ear artery smooth muscle cells. The current reversed near 0 mV, and the channels were permeable to both monovalent and divalent cations, but not to anions. In addition, Benham *et al.* (1987) occasionally observed a late outward current which they suggested was the result of activation of a Ca^{2+}-dependent K^+ conductance. There are, however, interesting differences among the ATP-activated currents in the three systems. Analysis of current fluctuations in chick muscle (Hume & Honig, 1986) and frog atrium (Friel & Bean, 1988) suggest that the ATP-activated channels have a very small single-channel conductance (< 1 pS), while single-channel recordings from ear artery (Benham & Tsien, 1987) demonstrate a channel with a unitary conductance of 20 pS when Na^+ is the charge carrier. Furthermore, the non-hydrolysable ATP analogue α,β-methylene ATP (AMP-CPP) is an agonist of the rabbit ear artery receptor (Benham & Tsien, 1987), an antagonist of the frog aortic receptor (Friel & Bean, 1988), and is without effect on the chick skeletal muscle receptor (Hume & Honig, 1986). At present, it is unclear whether these differences reflect variation among the species studied, or indicate that different subtypes of ATP receptors are found in different types of muscle.

With the knowledge gained by observing the action of ATP on myotubes bathed in non-physiological solutions, it is interesting to consider the effect that ATP has on muscle cells bathed in a normal extracellular solution. In mature myotubes, the resting potential is below −50 mV, so the predominant effect of ATP is to depolarize cells. Indeed, we have noted that most mature cells contract in response to ATP. However, the situation is likely to be quite different in developing fibres. Fischbach, Nameroff & Nelson (1971) reported that immature chick myoblasts have resting potentials in the range of −10 to −20 mV and that as cells grow they gradually acquire more negative resting potentials. Because of the difficulty of making high-quality intracellular recordings from small cells, this report was treated with scepticism by many investigators. However, we have confirmed these observations by making whole-cell recordings with patch clamp electrodes (unpublished

observations). As Fig. 4 demonstrated, cells with a resting potential in the range of −10 mV are hyperpolarized by ATP, primarily due to activation of the second phase. Thus, in order to understand whether the normal effect of ATP is to produce depolarization or hyperpolarization it will be crucial to learn when during development ATP is biologically active.

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